BIOACTIVATION OF CYSTEINE-S-CONJUGATES OF HALOGENATED ALKENES BY A NON-ENZYMIC MODEL FOR CYSTEINE CONJUGATE ß-LYASE

Jan N.M. Commandeur, Johnny Hageman, J. Thomas Sanderson, Luc Koymans and Nico P.E. Vermeulen. Department of Pharmacochemistry, Division of Molecular Toxicology, Free University, Amsterdam, the Netherlands.

Pyridoxal in the presence of certain metal ions (Cu(II), Fe(II), Fe(III), in borate buffer pH 8.6 acts as a cysteine conjugate ß-lyase-mimetic model, as indicated by time dependent formation of pyruvic acid from twelve cysteine (Cys) S-conjugates via ß-elimination; transamination was not observed. In presence of 4-(p-nitrobenzyl)pyridine (NBP), formation of alkylating reactive intermediates resulting from ß-elimination of nephrotoxic cysteine conjugates could be monitored directly by the formation of coloured adducts absorbing at 508-560 nm. The thiol-compounds formed from the non-toxic cysteine conjugates investigated did not alkylate NBP. An extremely high degree of NBP-alkylation was observed with the nephrotoxin and genotoxin S-1,2-dichlorovinyl-L-cysteine.

When analyzed by ^{19}F-NMR, S-(tetrafluoroethyl)-L-cysteine (TFE-Cys) was found to be converted to fluoride anion, and products derived from difluorothionoacyl fluoride: difluorothionoacetic acid, difluoroacetic acid and N-difluorothionoacetylated TFE-Cys. In incubations with S-(2-chloro-1,1,2-trifluorofluoroethyl)-L-cysteine (CTFE-Cys) and S-(2,2-dichloro-1,1-difluorofluoroethyl)-L-cysteine (DCDFE-Cys), formation of thionoacylated cysteine conjugates, was observed by GC-MS analysis, indicating formation of the corresponding thionoacyl fluoride compounds. However, chlorofluorothionoacyl fluoride-derived products amounted to only 10-15% of the amount of CTFE-Cys converted, suggesting the presence of an alternative rearrangement route. Formation of a thiirane, formed by elimination of the ß-chlorine atom, is proposed as the main route of rearrangement in the case of CTFE-Cys and DCDFE-Cys. ß-Elimination of S-(2,2-difluoroethyl)-L-cysteine (DFE-Cys), resulted in formation of a relatively stable thiol compound indicating a slow formation of a thiirane when only fluorine atoms are present at the ß-position of the S-ethyl-group. This is presumably due to the poor leaving group character of the fluoride anion.

Free enthalpies of formation of thiolates derived from TFE-Cys, CTFE-Cys and DCDFE-Cys, and the free enthalpies of formation of possible rearrangement products, thionoacyl fluorides and thiiranes, were calculated by ab initio calculations. For TFE-thiolate, formation of difluorothionoacyl fluoride is energetically favoured over formation of thiirane. In contrast, for CTFE-thiolate and DCDFE-thiolate the thiirane pathway was favoured over the thionoacyl fluoride pathway.

SODIUM NITRITE- AND SODIUM SULFITE-STIMULATED METABOLIC ACTIVATION OF BENZO(A)PYRENE-7,8-DIHYDRODIOL IN HUMAN POLYMORPHONUCLEAR LEUKOCYTES

D. Constantin, B. Jernström and P. Moldéus, Department of Toxicology, Karolinska Institutet, Box 60400, S-104 01 Stockholm, Sweden

Sodium nitrite and sodium sulfite (major products of inhaled NO_2 and SO_2) stimulated the metabolism of benzo(a)pyrene-7,8-dihydrodiol (BP-7,8-diol) in phorbol ester-activated human polymorphonuclear leukocytes (PMNs) to the reactive intermediate benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE), the ultimate carcinogenic metabolite of benzo(a)pyrene. The formation of BPDE from BP-7,8-diol metabolism was estimated through the measurement of the stable tetraols (7,8,9,10- and 7,10/8,9-tetrahydrotetraol) as well as protein and DNA binding. Both tetraol formation and the covalent binding to exogenous DNA and cellular proteins were significantly ($p<0.05$) enhanced in the presence of sodium nitrite. Sodium sulfite also increased the metabolism of BP-7,8-diol to tetraols but it had no effect on the metabolism of this enantiomer to protein- and DNA-binding metabolites. The stimulatory effect of nitrite or sulfite on the metabolism of BP-7,8-diol was only observed when the cells were stimulated with phorbol myristate acetate (PMA), a compound that induces oxygen activation in the PMNs.

The mechanisms by which the metabolism of BP-7,8-diol is stimulated by nitrite or sulfite has not yet been established but according to our results they seem to differ from each other. Thus PMA-stimulated PMNs catalyze the formation of a sulfite trioxide radical which could react with O_2 to form a peroxyfree radical, probably responsible for the epoxidation of BP-7,8-diol to its respective diol epoxide (BPDE). The sodium nitrite dependent activation of BP-7,8-diol in PMA-stimulated PMNs appears to be partly peroxidase dependent, mediated probably by myeloperoxidase, but other possible pathways involving a cyto-chrome P-450, lipooxygenase or NO, might participate in the nitrite dependent activation of BP-7,8-diol in leukocytes.

STUDY OF THE MUTAGENICITY OF 2-METHYLPROPENE (ISOBUTENE) AND ITS EPOXIDE IN THE AMES ASSAY, MODIFIED FOR VOLATILE COMPOUNDS.

M. Cornet, P. Castelain*, R. Laib**, A. Vercruysse and V. Rogiers.
Dept. of Toxicology, Vrije Universiteit Brussel, Laarbeeklaan 103, 1090 Brussels, Belgium; *Dept. of Antropogenetics, Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium; **Institut für Arbeitsphysiologie, Ardeystrasse 67, 4600 Dortmund, Germany.

In previous work, it has been demonstrated that 2-methylpropene (MP) or isobutene, a gaseous compound widely used in industry, is metabolized to the epoxide 2-methyl-1,2-epoxypropane (MEP). Epoxides are very reactive metabolites, known to be often responsible for mutagenic effects. Therefore, the mutagenic properties of MP and MEP were investigated using the Ames Salmonella assay, modified for volatile compounds. For this purpose, a simple exposure system consisting of gastight tissue culture flasks, has been used. This method has the advantage that the volatile test chemical is present during the entire incubation period and that several concentrations of the compound can be tested on one day.
Using this closed system, MP is not mutagenic in strains TA 100 and TA 102. MEP is mutagenic in strains TA 100, TA 102 and TA 1535, as demonstrated by a clear dose-response relationship. TA 1535 seems to be most sensitive to MEP in comparison with the other bacterial strains studied. For strain TA 1535, the mutagenic activity of MEP decreased significantly in the presence of S9-mix, indicating that the epoxide is inactivated by epoxide hydrolase and by glutathione S-transferase as reported previously.
From the present study it can be concluded, that very high concentrations of MEP are necessary to induce a mutagenic effect and that this epoxide can be detoxified by different liver enzymes.

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COMPARATIVE PHARMACOKINETICS OF RECOMBINANT SOLUBLE CD4 (ST4) IN RATS FOLLOWING SINGLE INTRAVENOUS, SUBCUTANEOUS, INTRAMUSCULAR, AND INTRAPERITONEAL ADMINISTRATION

Carl Crysler, James Urbanski, and Kei-Lai L. Fong, Department of Drug Metabolism and Pharmacokinetics, L-720, SmithKline Beecham Pharmaceuticals, P.O. Box 1539, King of Prussia, PA 19406, USA

CD4 is a glycoprotein receptor found predominantly on T lymphocytes, and is involved in recognition of class II major histocompatibility complex proteins, activation of immune responses, and acts as a receptor for the gp120 envelope glycoprotein of human immunodeficiency virus (HIV). A recombinant soluble form of CD4 (sT4, MW > 40 kd) has been shown to block the infectivity and cytopathic effects of HIV in vitro, and is currently being investigated for treatment of Acquired Immune Deficiency Syndrome (AIDS) and AIDS related complex (ARC). In order to explore alternative routes for chronic administration of sT4 and backup compounds (i.e., molecules that specifically block CD4-gp120 interactions) in animal models, the pharmacokinetics and bioavailability of sT4 were compared in male Sprague-Dawley rats following single intravenous (iv), subcutaneous (sc), intramuscular (im), and intraperitoneal (ip) administration at a dose of 0.3 mg/kg. Blood samples were collected through an indwelling catheter up to 72 hours post dose, and plasma sT4 concentrations were measured by a N-terminal gp120-binding domain specific Leu3A/pAb ELISA and by a N- and C-terminal specific Leu3A/OKT4 ELISA.

Similar results were obtained by either ELISA. Data is presented as mean ± SD. Following iv administration of sT4, plasma concentrations declined rapidly in a biexponential fashion with a $t_{1/2\alpha}$ of 7.21 ± 1.06 min and a $t_{1/2\beta}$ of 49 ± 6.9 min. The AUC in the alpha phase represented 93.0 ± 1.7% of the total AUC. Systemic plasma clearance was 8.4 ± 1.0 ml/min/kg and the volume of distribution at steady state was 111 ± 19 ml/kg.

sT4 was readily absorbed following either sc, im, or ip administration, with detectable plasma levels 5 to 10 minutes post injection. Estimated bioavailability for sc, im, and ip administrations were 20 ± 3.4%, 42 ± 16%, and 99 ± 26%, respectively (4 animals for each group). Cmax's were 17 ± 3.5, 57 ± 28, and 180 ± 77 ng/ml for sc, im, and ip routes, respectively, and plasma levels were detectable up to 12 hours post injection. Subsequent analysis of plasma samples by immunoaffinity isolation (Leu3A-Sepharose®) coupled with immunoblotting demonstrated that intact sT4 was the major immunoreactive species in plasma in all dosing groups. This result also demonstrates the validity of the Leu3A/pAb ELISA used in this pharmacokinetic study.

Conclusions: sT4 is rapidly absorbed and reaches systemic circulation as an intact molecule following either ip, im, or sc administration. Ip administration results in a higher Cmax and greater bioavailability than sc or im administration, and would be an efficient delivery method for in vivo efficacy evaluations in animal (particularly rodent) models.

STUDY ON THE METABOLISM OF A NEW ANTIARRHYTHMIC AGENT (BGYKI-38 233) IN RAT

Anita Csárdi, Gyula Horváth, Sándor Mészáros, Márta Pátfalusi, László Vereczkey, Institute for Drug Research 1325-Budapest, Hungary

BGYKI-38 233 (2,6-dimethylphenylhydrazo-N,N-dimethyl-formamidine-hydrochloride) is a new antiarrhythmic compound currently under clinical evaluation. Metabolic profile of BGYKI-38 233 in rat urine was studied. After a single 25 mg/kg oral dose of ^{14}C-labelled compound about 50% of dose was excreted in urine during 24 hours.

The original substance and its metabolites are unstable at neutral or alkaline pH-s and are sensitive to light. In order to supress oxidation urine samples have been collected in 0.1 N HCl solution, in a dark place. For determination of chemical degradation the ^{14}C-labelled compound was incubated in urine in vitro, under the same conditions.

The urine samples with or without enzymatic digestion were investigated with TLC and HPLC. After chromatographic separation the amounts of the components were measured by liquid scintillation counting. The radioactive components were identified by comparing their chromatographic properties to those of authentic standards. Further structural information was obtained from their mass spectra after isolation.

In rat urine besides the original compound hydroxylated derivatives (at positions 3 or 4 of aromatic ring) could be identified as real metabolites. The azo-derivatives proved to be degradation products formed in the isolation and purification procedures.

DISPOSITION OF METABOLICALLY LABELED TRITIATED SOLUBLE CD4 (ST4) IN RATS FOLLOWING INTRAVENOUS (IV) AND SUBCUTANEOUS (SC) ADMINISTRATION

Charles B. Davis, Kathleen E. Boyle, James Urbanski and Kei-Lai L. Fong, Department of Drug Metabolism and Pharmacokinetics, L720, SmithKline Beecham Pharmaceuticals, P.O. Box 1539, King of Prussia, PA, 19406, USA.

Recombinant soluble CD4 (MW 45,000) has been metabolically labeled with ^{3}H-leucine (the most abundant amino acid in the polypeptide) and shown to be chemically representative of the unlabeled molecule. To explore the biotransformation and disposition of a recombinant protein, this uniformly labeled ^{3}H-sT4 was administered intravenously and subcutaneously to Sprague-Dawley rats. Following a single dose of 0.3 mg/kg, blood samples were collected for 9 days and analyzed for total radioactivity, total plasma radioactivity, trichloroacetic acid precipitable plasma radioactivity, sT4 related plasma radioactivity (by extraction with a Sepharose bound polyclonal anti-sT4 antibody) and plasma sT4 concentration (by an N and C terminal specific Leu3A/OKT4 ELISA). Excreta were analyzed for total radioactivity.

The pharmacokinetic profiles of intact sT4 (based on the ELISA) were as expected from the results of previous studies. ST4 was eliminated rapidly from plasma with a $t_{1/2}$ of 6.6 min and clearance rate of 6.4 ml/min/kg. SC bioavailability was estimated to be < 15%. However, comparison of kinetic profiles of total radiolabel, TCA precipitable radiolabel, sT4 related radiolabel and the isolation of plasma proteins containing tritium have led to the following conclusions. (1) ST4 was degraded to its constituent amino acids and the labeled amino acids were subsequently incorporated into endogenous proteins. The plasma $t_{1/2}$ of non-sT4 related radiolabel was > 45 h. (2) Following subcutaneous administration, the sT4 was significantly degraded prior to reaching the vascular circulation. (3) Tritium was incorporated into blood cell proteins exhibiting a prolonged half-life ($t_{1/2}$ > 250 h). (4) Low urinary and fecal recovery of administered radioactivity over 9 days (< 9%) also suggested the possibility of incorporation of radiolabel into tissue protein.

PLASMA CONCENTRATION-RESPONSE RELATIONSHIP FOR INHIBITION BY CIPROFLOXACIN OF THEOPHYLLINE DISPOSITION

JOHN D DAVIS, BEVERLEY BRENNAN, LEON AARONS and J.BRIAN HOUSTON
Dept. of Pharmacy, University of Manchester, UK

Recent reports that ciprofloxacin (C) markedly affects the disposition of theophylline (T), suggest that C may inhibit the hepatic clearance of T. The effect of dosing rate on plasma clearance must be clearly defined, relative to other fluoroquinolones, in order to evaluate the degree and potency of the effect of C on T pharmacokinetics.

Using a rat model, we have designed suitable dosing regimes to achieve and maintain a range of steady state plasma concentrations of C. The development of a simple isocratic HPLC assay for the simultaneous detection of both T and C in the same sample has allowed us to monitor the plasma pharmacokinetics of both drugs concurrently. Single iv bolus doses of T were given to animals (n= 38) receiving constant iv infusions of C, to maintain concentrations (0.3 to 33.9 mgL^{-1}) set by bolus administration.

No differences were seen in the initial plasma concentration or volume of distribution for theophylline. There was a dose dependent reduction in total plasma clearance. The data were analysed using a model where CL is made up of two components:

$$CL = \frac{CL_1}{(1+\frac{C_{ss}}{K_I})} + CL_2$$

The model predicts that the K_I is 16.7 μM (6.4 mgL^{-1}) and that the value for unaffected clearance (CL_2) = 0.0128 Lhr^{-1}. It also predicts that the maximum effect of C on T clearance (CL_1) would be a fall of 0.0287 Lhr^{-1} (63% fall).

SELECTIVE DETECTION OF DIFFERENT $CHCl_3$ METABOLITES.

Angelo De Biasi, Simonetta Gemma, Emanuela Testai and Luciano Vittozzi.
Istituto Superiore di Sanità, Roma, Italy

Results of comparative studies with different strains of rodents suggested that the species-specificity of $CHCl_3$-induced hepatocarcinogenesis may be associated to the simultaneous presence of low-affinity oxidative and reductive pathways of chloroform bioactivation. Indeed, such a feature is typical only for B6C3F1 mice, the only strain developing hepatic tumors.

Since microsomal phospholipids (PL) are a good target of chloroform metabolites produced both oxidatively and reductively, a method based on PL hydrolysis has been developed to detect selectively $CHCl_3$ metabolites.

The radioactivity associated with the oxidation intermediates bound to PL partitions preferentially in the aqueous phase, whereas the radioactivity derived from the reduction intermediates is localized almost completely in the organic phase.

As suggested by our results, the reactive oxidative and reductive intermediates ($COCl_2$ and $CHCl_2$, respectively) show a typical regioselectivity in the attack to PL.

Since PL are suggested to be the major site of chloroform damage, this feature may be very useful to study selectively the different roles played by the oxidative and reductive metabolites in the toxicity of this compound.

EFFECT OF DOSE LEVEL AND MULTIPLE DOSING ON THE PHARMACOKINETICS OF IDAVERINE IN THE DOG

N. DE LANGE, DUPHAR BV, PO BOX 900, 1380 DA, WEESP, THE NETHERLANDS.

IDAVERINE, a product of DUPHAR research, has demonstrated a potent and long lasting antispasmodic action in animals.

Four pharmacokinetic studies were executed in both male and female dogs.

1 Dose dependency after intravenous administration of 1 and 5 $mg.kg^{-1}$
2 Dose dependency after oral administration of 0.2, 1, 5 and 12 $mg.kg^{-1}$
3 Effect of multiple daily oral dosing of 5 $mg.kg^{-1}$ for six days on the parameters calculated after oral dosing.
4 Effect of multiple oral daily dosing of 5 $mg.kg^{-1}$ on the parameters calculated after intravenous dosing.

The results show:
AUC after correction for the dose level after intravenous administration of 5 $mg.kg^{-1}$ was 54% larger than after a dose of 1 $mg.kg^{-1}$. Distribution volume at steady state was for both doses about 5 $l.kg^{-1}$, elimination half-life about 1 hour. Total body clearance was 5 $l.kg^{-1}.h^{-1}$ and 3 $l.kg^{-1}.h^{-1}$ for respectively a dose of 1 $mg.kg^{-1}$ and 5 $mg.kg^{-1}$.
AUC's after multiple oral administration of 5 $mg.kg^{-1}$ were more than twice the AUC's after single administration of 5 $mg.kg^{-1}$.
Dose corrected AUCs after oral administration normalized to a dose of 1 $mg.kg^{-1}$ ranged from 9 $ng.ml^{-1}.h$ for a dose of 0.2 $mg.kg^{-1}$ to 300 $ng.ml^{-1}.h$ for a dose of 12 $mg.kg^{-1}$.
After intravenous administration of 5 $mg.kg^{-1}$ preceded by six daily oral doses of 5 $mg.kg^{-1}$ AUCs were 60% higher in comparison to dogs not receiving oral doses.

In none of the experiments effect of dose level or multiple dosing was seen on the distribution volume or terminal half-life.

Conclusion:
Total body clearance decreased at higher dose and also after multiple dosing.

IS THE SCOPARONE O-DEMETHYLASE ASSAY USEFUL FOR CATEGORIZING TYPES OF HEPATIC MICROSOMAL MONOOXYGENASE INDUCERS ?

Jean-P. Desager, Yves Horsmans, Carl Harvengt. Université Catholique de Louvain, B-1200 Brussels-Belgium.

Scoparone (6,7 dimethoxycoumarin) is demethylated to scopoletin (7-OH-6-methoxycoumarin) and isoscopoletin (6-OH-7-methoxycoumarin) by the cytochrome P-450 monooxygenase system. The ratio of scopoletin to isoscopoletin has been proposed to distinguish between a 3-methylcholantrene (3-MC)-like or phenobarbital (PB)-like induction in rat liver microsomes and in mice.
The aim of this study was to investigate the value of this ratio in guinea pigs liver microsomes.
Method. Liver microsomes from untreated and from 3-MC-, PB- and ARL-115-pretreated Dunkin-Hartley guinea pigs were isolated.
Liver microsomes from untreated and 3-MC-, PB- and ARL-115-pretreated Sprague Dawley rats were also isolated to check the already published results.
The HPLC method described by Müller-Enoch et al. (1) was used to determine the two known metabolites of scoparone.
Results. The product ratio scopoletin : isoscopoletin was between 1:0.20 and 1:0.26 in untreated as well as in all pretreated guinea pigs. The different inducers were responsible for an elevation of an absolute amount of the two metabolites without change in the ratio of the demethylation products. At the opposite, the ratios were 1:2.3 in untreated and in 3-MC-pretreated rats whereas the ratio was reversed (1:0.26) in PB-pretreated rats as published. Ratios obtained in ARL-115-pretreated animals showed also a clear dissociation between the two species: 1:0.26 in guinea pigs and 1:3.7 in rats.
Conclusions. The scoparone O-demethylase assay cannot be used to differentiate a 3-MC induction from a PB induction in guinea pigs.
These data stress attention on the difficulties to extrapolate for cytochrome P-450 activities from one species to another one.

(1) D.Müller-Enoch et al. Arzneim. Forsch. 38, 1520-1522 (1988).

EFFECTS OF SEVERAL INDUCERS ON CYTOCHROME P450 IIB1/2, IIE1 AND IIIA EXPRESSION IN A RAT HEPATOMA CELL LINE (FaO).

de Waziers, I.[a], Bouguet, J.[b], Barouki, R.[b] and Beaune, P.H.[a]

[a] INSERM U75, 156 rue de Vaugirard, 75730 Paris Cedex 15, France
[b] INSERM U99, Hôpital Henri Mondor, 94010 Créteil, France.

Cytochromes P 450 belong to a superfamily of inducible enzymes involved in the metabolism of endogenous and exogenous substrates. The regulation of the expression of these enzymes still remains unclear and several models have been developped to carry out genetic and biochemical studies. Among them, the FaO cells, derived from rat Reuber H 35 hepatoma, constitutes a suitable model. The aim of our study was to analyse the effects of ethanol (E), dexamethasone (D) and RU 486 (RU), a glucocorticoid and a progesterone antagonist, on the expression of several cytochromes P 450 in FaO cells.

FaO cells were treated during 24 or 48 hours with the following inducers: E (140 mM), D (10^{-7}M), RU (5.10^{-7}M), E+D or RU+D. Specific RNAs were measured by Northern blots hybridized with the following probes: P 450 IIB1, IIE1, IIIA and actin. Proteins were measured by Western blots probed with specific antibodies directed against P450 IIB1/B2, IIE1 and IIIA1/A2.

All our results were compared to those obtained in control cells without treatment. E increased, from 2 to 4-fold, the amounts of P450 IIB and IIE mRNA but did not modify the P 450 IIIA mRNA level. D decreased the amounts of P 450 IIB and IIE mRNA and antagonized the inducing effect of E on these P 450. Ru did not induce important modifications in the amounts of the three P 450 mRNA but Ru+D decreased them considerably. All the effects observed at 24h are amplified at 48h. Whatever the treatment, protein concentrations of each P450 studied were correlated with the corresponding amounts of mRNA.

The present study clearly shows that cytochromes P 450 are under distinctly different regulatory control. Therefore, the FaO cells appear to provide a suitable system to analyse genetic and molecular factors controlling the expression of rat P 450 genes.

THE USE OF HUMAN HEPATOCYTES TO PREDICT STEREOSELECTIVE METABOLISM IN MAN.

Maurice Dickins[a], Tim Morley[a], Mark Hadley[b], Tony Padfield[b], and John Salmon[b], Departments of Drug Safety Evaluation[a] and Pharmacology[b], Wellcome Research Laboratories, Beckenham, Kent. U.K.

Inhibition of arachidonate 5-lipoxygenase (5-LO) is a potential target for therapeutic intervention in asthma. Acetohydroxamic acids such as BW A4C are potent and selective inhibitors in vitro and also inhibit 5-LO activity in vivo following oral administration. However, BW A4C was found to be extensively metabolised in man and more stable molecules were required. More recently, a series of hydroxyureas which are also potent 5-LO inhibitors have been synthesised and subjected to metabolic studies. In vivo data from rats and dogs suggested that the chiral hydroxyurea BW B70C was more metabolically stable than BW A4C in these species. Incubations using isolated hepatocytes showed that liver cells from both rats and dogs could satisfactorily mimic the metabolism of BW B70C. Furthermore, stereoselective differences in metabolism of the R and S enantiomers of BW B70C were particularly marked in the dog but were much less apparent in the rat. This species difference in the metabolism of the enantiomers of BW B70C seen in vivo could be reproduced in vitro using hepatocytes from rats and dogs. Thus human hepatocytes were used as a model system for man in an attempt to predict in vivo metabolism. As was the case in vivo, BW A4C was extensively metabolised by human hepatocytes to the metabolites found following oral administration of BW A4C to man. Similar experiments have been carried out for BW B70C and its individual enantiomers using human liver cells and the results have been used to predict the stereochemical metabolism of these molecules in man. These data will be used in part to select the most appropriate enantiomer for future drug development.

THE GSH PROTECTION AGAINST THE MERCURIC CHLORIDE TOXICITY IN VARIOUS ORGANS IN MOUSE: EFFECT ON GSH-DEPENDENT ENZYMES.

Paolo Di Simplicio, Rita Vignani and Massimo Gorelli, Dept. of Environmental Biology, University of Siena, Italy.

The effect of $HgCl_2$ and $HgCl_2$+GSH administration on various GSH-dependent enzymes in different mouse organs was studied. After 3 days of treatment with 100 umoles/Kg per os, severe symptoms of toxicity were observed, whereas in mice receiving GSH+$HgCl_2$ (2:1) this was found on the 4th day. GSH-transferase GSH-peroxidase, GSSG-reductase, glyoxalase I and II, thiol-transferase, γ-glutamyltraspeptidase (GT) and γ-glutamylcysteine synthetase (SYNT) activities were assayed in the cytosol of various organs. $HgCl_2$ caused a decrease in all activities in liver and kidney, with the exclusion of GT and SYNT in liver. Small variations were in general observed in lung, muscle and brain. The majority of intestine enzymes were increased in their activities. In GSH protected animals the liver and kidney activities returned to control values, whereas those of intestine were not further increased except in few cases. In the remaining organs small variations were seen with few exceptions. The GSH content decreased in liver (-60%) kidney and muscle (about -40%), lung (-25%) and intestine (-15%) in $HgCl_2$ mice and returned to controls in protected ones.

INDUCTION OF CYTOCHROME P-450 IVA1 IN THE LIVER OF RATS TREATED WITH PEROXISOME PROLIFERATING COMPOUNDS AND CLASSICAL P-450 INDUCERS.

H.A.A.M. Dirven, P.H.H. van den Broek, J.G.P. Peters, J. Noordhoek and F.J. Jongeneelen. Department of Toxicology, University of Nijmegen, P.O. box 9101, 6500 HB Nijmegen, The Netherlands.

Peroxisome proliferating compounds are a structurally diverse group of chemicals, whose common feature is the ability to induce proliferation of peroxisomes in the liver. The carcinogenicity of a compound from this group is associated with its potency of induction of peroxisomes in rodents[1]. It has been suggested that the proliferation of peroxisomes is the result of a perturbation of fatty acid metabolism in hepatocytes which is the result of induction of cytochrome P-450 IVA1[2].

We have treated rats orally with the plasticizer di-(2-ethylhexyl)phthalate (DEHP). At dose-levels of 50, 100 and 250 mg DEHP/kg body weight per day no changes in total cytochrome P-450 values were found, while cytochrome P-450 IVA1 was 2-4 times induced. Lauric acid hydroxylase activity (marker for cytochrome P450 IVA1) was measured with a newly developed assay without the need of radiolabeled substrates[3]. The lauric acid omega-hydroxylase activity showed a dose-dependent increase, while no increase in lauric acid (omega-1)-hydroxylase activity was found. A strong correlation between the induction of cytochrome P-450 IVA1 and the activity of peroxisomal palmitoyl-CoA oxidase and carnitine acetyltransferase was found.

We also treated rats with the DEHP metabolites mono-(2-ethylhexyl)phthalate, 2-ethyl-1-hexanol and 2-ethyl-1-hexanoic acid. These compounds also induced cytochrome P-450 IVA1, lauric acid omega-hydroxylase activity and palmitoyl-CoA oxidase activity.

In contrast, in rats treated with known cytochrome P-450 inducers like aroclor-1254, phenobarbital and 3-methylcholanthrene, no induction of lauric acid omega-hydroxylase activity, lauric acid (omega-1)-hydroxylase activity or immunochemically determined cytochrome P-450 IVA1 was found.

These data further strengthen the relationship between cytochrome P-450 IVA1 induction and peroxisome proliferation. Induction of cytochrome P-450 IVA1 by xenobiotics appears to be limited to compounds which have a peroxisome proliferating capacity. We postulate that species differences in peroxisome proliferation are due to interspecies differences in the inducibility of cytochrome P-450 IVA1. Recently, we have determined a pronounced lauric acid omega-hydroxylase activity in microsomal fractions of monkey and human liver. In these species we have also found proteins immunochemically related to cytochrome P-450 IVA1 (submitted). Species differences in the inducibility of these enzymes will be studied with the use of primary cultures of hepatocytes of rodents and primates.

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MICROSOMAL METABOLISM OF THIOCARBAMIDES: ROLE OF FLAVIN-CONTAINING AND P450 MONOOXYGENASES.

Daniel R. Doerge and Caroline J. Decker, Univ. Hawaii, Honolulu, HI 96822

The oxidative metabolism of thiocarbamides was investigated in rat liver microsomes using the EBDC fungicide metabolite, ethylenethiourea (ETU), and model anti-hyperthyroid drugs based on benzimidazole-2-thione (MBI). Microsomal metabolism of thiocarbamides results in the destruction of P450 chromophore and loss of P450 isozyme activity via covalent binding to protein thiols. These effects are totally abolished by coincubation with physiological concentrations of GSH. Heat pretreatment of microsomes to selectively inactivate the flavin-containing monooxygenase (FMO) attenuates ETU- and MBI-induced chromophore loss, binding and P450 activity loss suggesting a predominant role for FMO in the production of reactive, diffusible metabolites. However, these metabolites appear incapable of covalent binding to exogenous single stranded DNA. Use of the selective P450 suicide substrate, 1-aminobenzotriazole, showed that some P450 isozymes contribute to the binding of thiocarbamides to microsomal proteins. The binding of ^{35}S- and ^{14}C-labelled thiocarbamides to microsomal proteins was identical whether catalyzed by FMO or P450 suggesting that production of reactive fragments does not occur. These results are consistent with the hypothesis that FMO and P450 catalyze S-oxygenation of thiocarbamides to produce reactive sulfenic acids which form disulfide adducts with microsomal proteins.

ENANTIOMERIC METABOLIC DIFFERENCES DETECTED USING *IN VITRO* AND *IN VIVO* TECHNIQUES

J. Neil Duncan, Phillip Jeffrey, Susan A. Rees and Don J. Nichols. Upjohn Laboratories - UK, Fleming Way, Crawley. RH10 2NJ. UK.

A group of aminotetralins (a series of compounds related to 8OH-DPAT) are currently under investigation in our laboratories. Although pharmacologically active, they are subject to extensive first-pass metabolism. In an effort to select compounds with metabolic stability, a two tier screening process based on clearance has been developed.

Rat hepatocytes are prepared by a modification of the collagenase perfusion method (1). *In vitro* clearance is assessed by incubating the compound at three concentrations in suspensions of freshly prepared rat hepatocytes (5 million cells ml^{-1}) to obtain a measure of their metabolic stability relative to a standard compound. *In vivo* clearance (CL) in rats is determined for the most metabolically stable compounds *in vitro*, using a simple intravenous infusion technique. The compound is infused to steady state (12 h) and two blood samples (11.5 and 12 h) are removed for analysis by HPLC. CL = infusion rate/C_{ss} where C_{ss} is the steady state concentration.

Using the above methodology, enantiomeric differences in *in vitro* metabolic stability and *in vivo* plasma clearance have been noted for several pairs of enantiomers. In each case, the greater metabolic stability *in vitro* was reflected in reduced plasma clearances *in vivo*. This is important since it provides confidence in the correlation between *in vitro* and *in vivo* data for this series of compounds and justifies the use of hepatocyte data in drug candidate selection.

(1) Reese JA and Byard JL. (1981). *In vitro*. 17. 935-940.

REGULATION OF CYTOCHROME P-450IIB1 GENE EXPRESSION

Michael Ediqkaufer, Pablo Steinberg, Franz Oesch, Johannes Doehmer. Institut für Toxikologie, J. Gutenberg-Universität Mainz, Obere Zahlbacher Str. 63, D-6500 Mainz, F.R.G.

Phenobarbital (PB) is a potent inducer of cytochrome P-450IIB1 in rat liver. Increased Cyt.P-450IIB1 activity is due to an increased transcription level. However, there is only little information concerning the molecular basis of Cyt.P-450IIB1 gene regulation.
A λ-DASH rat liver EcoRI genomic library was constructed and screened with a random primed P-450IIB1 probe obtained from the first exon. Three recombinant phages were detected. The Cyt.P-450IIB1 0.6 kb 5´-flanking region was isolated and linked to the Cyt.P-450IIB1 cDNA. The promotor area of this "minigene" (pP600-P450IIB1) was confirmed by sequencing. The "minigene" was cointroduced with the neomycin resistant gene as selection marker into HPRT- V79 Chinese fibroblasts. G-419 resistant clones were selected and characterized by Southern and Western-blotting. Four of these clones (ME1,2,3,4) were found to express Cyt.P-450IIB1. Treatment of these cells with PB ($2x10^{-3}$ and 10^{-6} M respectively, 72h) does not appear to affect the transcription level.
Another cell line containing a "minigene" with 375 bp 5´-flanking region (pP375-P450IIB1) showed neither basal expression nor inducibility. To investigate the role of the deleted 233 bp region an *in vitro* transcription system was set up. Only pP600-P450IIB1 was transcribed using rat liver whole cell extracts from control animals. Only the transcription level of pP600-P450IIB1 was increased by using whole cell extracts from PB treated rats. Gel-shift assays with the 233 bp fragment and two fragments derived from the 375 bp 5´-flanking region showed band shifting. Taken together we conclude that the Cyt.P450IIB1 promotor is under multiple control and even a fragment located 375 bp upstream of the transcription starting point is essential for gene expression.

THE UPTAKE AND METABOLIC EFFECTS OF INHALED 1,1,1-TRIHALOETHANES

Nagi I. Eldirdiri, George Loizou and Laurence J. King,Toxicology Division, School of Biological Sciences, University of Surrey, Guildford, U .K.

Rats were exposed to different concentrations of CH_3CCl_3, CH_3CCl_2F, CH_3CClF_2 and CH_3CF_3 by inhalation in a closed recirculating atmosphere exposure system. The depletion rate of chemicals from the atmosphere was monitored as uptake and metabolism proceeded. After the exposure, the concentrations of total glutathione (GSH) and glutathione disulphide (GSSG) in liver and lung, and selected serum enzyme activities were determined. The results were correlated with the physico-chemical properties of the compounds - molar polarizability (α mol), activation energy (Δ E), and partition coefficient (log P).

The rate of uptake decreased with increased fluorine content, and correlated directly with log P and α mol, and inversely with ΔE. All four compounds depleted total GSH in liver, but not in lung; GSSG was unchanged. The exposure concentrations required to produce GSH depletion increased with increasing fluorine substitution, possibly reflecting the dependence of uptake on log P. No changes were seen in serum enzymes up to 24h after exposure.

The binding affinity for hepatic P-450 (Ks) was greatest with CH_3CCl_3, and Ks values for the series varied inversely with log P. There was no relationship between binding affinity and GSH depletion, the latter appearing to relate to the total body burden of the compounds.

COMPARISON OF KETOCONAZOLE AND FLUCONAZOLE AS INHIBITORS OF HEPATIC CYTOCHROME P450 ACTIVITY.

C.M.ERVINE, B.BRENNAN, S.JEZEQUEL, M.J.HUMPHREY, and J.B.HOUSTON
Dept. of Pharmacy, University of Manchester, UK

The antifungal agents Ketoconazole (K) and Fluconazole (F) are believed to act via the inhibition of Cytochrome P450 mediated synthesis of the fungal cell membrane , Ergosterol. Both have also been shown to inhibit mammalian hepatic Cytochrome P450, but to differing degrees.

The purpose of the present study is to quantify this difference by means of a steady state plasma concentration-inhibitory responce study where both inhibitor and probe drug are assayed. Antipyrine elimination was determined, by HPLC, in the presence of a range of steady state K or F plasma concentrations in the male Sprague Dawley rat (ranges 2.3-83μM and 0.03-152μM, n=31 and 34, for K and F respectively).

The data was analysed using the model:

$$CL = CL_1/(1 + C_{SS}/Ki) + CL_2$$

where CL_1 and CL_2 are the antipyrine clearances affected and unaffected, by the inhibitor, respectively.The Ki values for K and F were found to be 3.1μM and 9.5μM respectively. This difference is increased further when results are expressed in terms of plasma concentration of unbound drug. The potency difference is 73 fold, in contrast to their similar inhibitory activity seen for the demethylation of Lanosterol by Candida Albicans.

Physiologically Based Pharmacokinetic Modelling of 1,3-Butadiene: Increased Importance of Lung Metabolism at Low Doses.

C.T.A. Evelo[1], N.G.M. Palmen[1], W.F. ten Berge[2] and P.J.A. Borm[1].
1: Department of Occupational & Environmental Health and Toxicology, University of Limburg, Maastricht, The Netherlands.
2: DSM, Corporate Department of Safety and Environment, Geleen.

The carcinogenicity of butadiene is attributed to target organ levels of the reactive metabolite 1,2-epoxybutene-3 (butadiene monoxide). A physiologically based pharmacokinetic model for 1,3-butadiene uptake, distribution and metabolic clearance was developed. Butadiene monoxidase activity was accounted for by inclusion of Michaelis Menten type reaction descriptors for the alveolar and the bronchiolar parts of the lung and for the liver. The model was validated with literature data from closed chamber studies with rats and mice by Kreiling *et al* and Bolt *et al*. The decrease of the butadiene concentration and the kinetic parameters calculated thereoff were found to fit closely.

The rate of metabolite formation was calculated for the liver and the alveolar and bronchiolar parts of the lung. For mice the metabolism in the alveolar part, where butadiene concentrations are highest, was found to become saturated at ambient air concentrations as low as 2 ppm i.e. well below the current dutch TLV of 50 ppm. Since metabolism in the other compartments did not become saturated before much higher concentrations (over 100 ppm) were reached, the relative importance of (alveolar) lung metabolism decreased with increasing exposure concentrations.

This study demonstrates the value of PB-PK models in extrapolation from high dose experimental studies to the lower exposures occuring in practice. Since the actual concentrations where saturation occurs is determined by species dependent parameters, this type of PB-PK model can also be used for a correct evaluation of interspecies differences.

[1] Bolt H.M., J.G. Filser and F. Störmer (1984) *Arch. Toxicol.* **55**: 213-218.
[2] Kreiling R., R.J. Laib, J.G. Filser and H.M. Bolt (1986) *Arch. Toxicol.* **58**: 235-238.

THE METABOLISM AND DISPOSITION OF EFLUMAST (RP42068) IN THE RAT

V. Facchini, G.D.Jones, N.E. Wilsher and P.P. Joannou; Biopharmacy R&D, Rhône-Poulenc Rorer Ltd., Dagenham Research Centre, Essex, U.K..

The metabolism and disposition of [14C] eflumast (RP42068), an anti-allergic agent, has been studied after single oral administration to rats. The product was well absorbed, with subsequent excretion of radioactivity occurring predominantly in urine (71.1%), to a lesser degree in faeces (23.3%), while only negligible levels were detected in expired air (<0.1%).

Unchanged parent compound was the sole product determined in post administration plasma and the major excretory product in both urine and faeces; constituting respectively 70 and 50% of radioactivity therein, although a number of metabolites were also detected. Three urinary metabolites were isolated and tentatively identified as an O-glucuronide and two different N-glucuronides.

Peak systemic levels were noted at 12 hours, with highest tissues levels predominantly evident 4-12 hours post dose. Other than gastro-intestinal tissue the highest concentration was determined in the target organ, the lung (0.93 µg equivalents/g). Tissue distribution revealed no marked retention, although low levels (<0.2 µg equivalents/g) were seen to persist up to 72 hours post dose in certain non gastro-intestinal tract tissues.

Whole body autoradiography failed to reveal significant differences in the distribution of radioactivity between sexes, or any evidence of binding to melanin-containing tissues.

THE METABOLISM OF HALOPERIDOL AND ITS IMPLICATION FOR IATROGENIC DISEASE

Jian FANG & John W. GORROD Chelsea Department of Pharmacy, King's College, University of London, Manresa Road, London SW3 6LX, UK.

Haloperidol (HAL) is among the most potent and widely used neuroleptic drugs in current clinical practice. In a study on the metabolism of HAL using hepatic preparations from various animal species, dehydroxyhaloperidol-pyridinium ion (DHHPI) was identified as a microsomal metabolite of HAL. This metabolic pathway is analogous with that observed during the activation of the neurotoxin N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) to N-methyl-4-phenylpyridine (MPP^+). MPTP was shown to induce a syndrome similar to Parkinson's disease in man and experimental animals. Subsequently, dehydroxy-3,4-dehydro-haloperidol (DHDHH), a closer analogue of MPTP, was shown to be one of the intermediate metabolites in the conversion of HAL to DHHPI. DHDHH was also converted to 4-(4-chlorophenyl)-1,2,3,6-tetrahydropyridine and dehydroxy-3,4-dehydro-haloperidol N-oxide when incubated with NADPH fortified microsomal preparations. All the metabolites were identical to the synthetic compounds in UV spectra and chromatographic characteristics. Dehydration of a tertiary alcohol to form a double bond, as occurs in the conversion of HAL to DHDHH, probably represent a new metabolic route. The striking similarities of the metabolic pathways between HAL and MPTP may throw new light on the cause of motor neuron side effects produced by HAL.

Additionally, 4-(4-chlorophenyl)-4-hydroxypiperidine was identified as a metabolite of HAL.

The probable role of other intermediates and metabolites as well as the enzymology involved in the metabolism of HAL will be presented. A brief survey of the toxicology of the metabolites as related to MPTP will also be reported.

I.V. ADMINISTRATION OF [^{14}C]-CD 271 IN THE RAT: METABOLITE CARACTERISATION DURING ENTEROHEPATIC CIRCULATION.

C. FILAQUIER, M.H. PEIRONE, P. BUCHAN, E. LAMAUD, #P. DUCHENE, CIRD GALDERMA, Sophia Antipolis, 06565 Valbonne, #ADME Bioanalyses, Sophia Antipolis, 06251 Mougins.

The metabolism of CD 271, a synthetic retinoid developped for the topical treatment of acne, was investigated during enterohepatic circulation in the male rat. After an I.V. bolus of [^{14}C]-CD 271 (6-[3-(1-Adamantyl)-4-methoxyphenyl]-2-naphthoic acid) in the rat (0.5 mg/ 90 µCi/ kg) 79 % of the administered radioactivity was excreted in the bile collected in the first 24 hours. Following intraduodenal slow infusion of 3-6 hour bile sample (period of maximum biliary excretion) 49 % of radioactivity was recovered in the bile within 48 hours, indicating a significant enterohepatic circulation.

Metabolic profile of "**Biliary excretion**" and "**Enterohepatic circulation**" samples were examined by gradient elution over an HPLC reversed phase column equipped with on-line detection of radioactivity. Specific hydrolysis permitted the estimation of conjugation reactions involved for different metabolite fractions.

Analysis of "**Biliary excretion**" samples revealed a major metabolite M2, as a glucuro-conjugate. The other metabolites detected were M6 and M7 principally in glucurono- and sulphoconjugated form, and in order of decreasing percentage M4, M5 and M0/M1 the most polar compounds. The parent drug CD 271 (M8) was not detected in free form during the first 24 hours of biliary excretion, but only as the glucuronide and the sulphoconjugate. In summary, during biliary excretion following I.V. administration of[^{14}C]-CD 271 the total metabolic pool was largely conjugated, 63.2 % as glucuronides, 17.1 % as sulphoconjugates and only 19.1 % was formed in the free form.

In "**Enterohepatic circulation**" samples parent drug CD 271 (M8) and M7 were not detected, this could be explained by either reabsorption followed by an intense Phase I metabolism, or a possible lack of absorption followed by direct elimination in faeces. The main metabolite remained the fraction M2 greater in the free form, and in order of decreasing percentage M6, M4, and M0/M1 were detected. After enterohepatic circulation the majority of the metabolic pool was in the free form, 75 % of the total radioactivity. 24.1 % of the metabolic pool appeared in glucuronide forms and the sulpho-conjugation was almost negligible.

CAFFEINE AND THEOPHYLLINE BIOTRANSFORMATION IN CELLS EXPRESSING A SINGLE CYTOCHROME P450 ISOZYME

U.Fuhr, J.Doehmer*, C.Janssens*, F.Oesch*, T.Kudla, Y.Keita, A.H.Staib, Dept. of Clinical Pharmacology, University Hospital Frankfurt, 6000 Frankfurt/Main 70; *Institute of Toxicology, University Mainz, 6500 Mainz; F.R.G.

Primary steps in the metabolism of caffeine (i.e. 1,3,7-trimethylxanthine (137X)) and theophylline (13X) are cleavage of methyl groups or hydroxalytion at pos. 8, mediated by cytochromes P450. To overcome problems arising in proper allocation of pathways to isozymes by conventional techniques (e.g. isozyme purification, antibody and/or inhibitor specifity), we used V79 chinese hamster cells which have been genetically engineered for stable expression of rat cytochromes P450IA1 (XEM2), P450IA2 (XEMdMz), or P450IIB1 (SD1), and controls (V79). 137X and/or 13X (4 mM) were incubated for 3 days with each of the cell lines. Concentrations of metabolites formed were determined in the incubation medium by HPLC (table):

Incubated with:	CAFFEINE(137X)				THEOPHYLLINE(13X)		
Metabolite (uM):	13X	17X	37X	137U	1X	3X	13U
XEM2 (P450IA1)	0.2	<0.3	<0.2	0.2	<0.5	0.3	6.4
XEMdMz (P450IA2)	1.1	2.2	1.9	0.6	<0.5	0.6	4.9
SD1 (P450IIB1)	<0.2	<0.3	<0.2	0.2	<0.5	<0.1	0.7
V79 (no P450)	<0.2	<0.3	<0.2	<0.1	<0.5	<0.1	<0.5

Thus, rat P450IA2 rather than P450IA1 is responsible for primary caffeine degradation, demethylation contributing 9fold more than 8-hydroxylation to the first biodegradation steps. This is consistent to investigations using rat liver microsomes. Therefore summarized caffeine degradation may be used to monitor P450IA2 activity. Theophylline was metabolized by both P450IA1 and IA2, 8-hydroxylation exceeding activity of demethylation several times.

METABOLIC DISPOSITION OF EBASTINE IN RATS

T. Fujii, T. Hatoyama, S. Matsumoto, H. Amejima, K. Yoshida, H. Miyazaki and M. Hashimoto, Research Laboratories, Dainippon Pharmaceutical Co.,Ltd., Osaka, Japan

Ebastine, a new H_1-histamine receptor antagonist, is converted to carebastine, a carboxilic acid metabolite, which is an actual active principle. Disposition of ebastine was studied in rats for clarifying the major site of biotransformation to carebastine and the additional metabolic pathway, if any.
Experiments in pylorus-ligated and intestine-looped rats revealed that ebastine was mainly absorbed from the small intestine. At 1 hour after oral administration of [^{14}C]ebastine, small intestine contained a small amount of unchanged drug and a large amount of carebastine. In situ study revealed that carebastine was the major radioactive material with minor component of possible intermediate (hydroxymethyl-metabolite) in the portal blood circulated from an isolated small intestine loop where the drug had been administerd. Ebastine was also metabolized to carebastine in 9,000xg sup of the liver. In rats, excretion of radioactive materials was predominant in fecal pathway. Identification of fecal metabolites revealed that ebastine was metabolized to carebastine via the hydroxymethyl intermediate and further, they underwent hydroxylation at their benzene moiety and sulfate conjugation.
These results indicate that ebastine was metabolized to carebastine mainly in the small intestine after oral administration and to additional metabolites possibly in the liver.

STEREOSELECTIVE TISSUE DISTRIBUTION OF CARVEDILOL ENANTIOMERS IN RATS

M. Fujimaki, Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan

After bolus i.v. injection of racemic carvedilol of 2 mg/kg to the rat, the R(+)- and S(-)-enantiomer levels in the blood and tissues (liver, kidney, heart, muscle, aorta) were measured by the stereospecific HPLC assay to clarify whether the two enantiomers exhibit a stereoselective tissue distribution. The blood levels of the two enantiomers declined in a biphasic manner but not parallel; the blood level of the R(+) was higher than that of the S(-) until 45 min after dosing, with the maximum R/S enantiomer ratio of 1.47 at 15 min, whereas after 90 min the R(+) was lower than that of the S(-). As compared with the R(+), the S(-) has a larger Vdss (3.32 vs. 2.21 l/kg), MRT (33.4 vs. 25.6 min) and CLtot (96.1 vs. 83.8 ml/min/kg). The tissue-to-blood partition coefficient values for the S(-) were 1.5 to 2.1-fold larger than those for the R(+) in all tissues, showing that the S(-) distributes more extensively to the tissues. These results were consistent with the greater Vdss for the S(-) estimated from systemic blood data. The values of the free fraction in tissue (fT) for the two enantiomers in the heart and muscle were nearly identical, but the fT values for the S(-) in the liver and kidney were slightly smaller than those for the R(+). The free fraction in blood for the S(-) was 1.65-fold larger than that for the R(+). These data suggested that the stereoselective tissue distribution of carvedilol enantiomers could be ascribed to the enantiomeric difference in plasma protein binding rather than in tissue binding.

IN VITRO METABOLISM OF DM-9384 USING RAT, DOG AND MONKEY LIVER MICROSOMES

Y. Fujimaki, K. Sudo and H. Hakusui, Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan

DM-9384 (N-(2,6-dimethylphenyl)-2-(2-oxo-1-pyrrolidinyl) acetamide, DM) is a novel cognition-enhancing agent. In previous in vivo studies, we have shown that DM was biotransformed to various oxidative metabolites and excreted mainly into the urine. In order to assess the hepatic metabolism, in vitro metabolism of DM was studied using the microsomes prepared from the liver from 3 species: rats (male and female), dogs and monkeys. After 30 min incubation of rat microsomes with DM, six metabolites were detected in the sample by reversed-phase HPLC. Those were identified by thermospray LC/MS/MS as 3-, 4- or 5-hydroxylated metabolites of the pyrrolizine ring moiety (M-3, M-20 and M-5), methylhydroxylated metabolite (M-4), and 3- or 4- hydroxylated metabolites of the aromatic ring moiety (M-18 and M-6). The microsomal metabolism of DM in various species produced qualitatively similar metabolic profiles. However, quantitative differences were observed. The major metabolite with all species was M-3. The other metabolites were minor, except for M-4 in male rats and M-18 in dogs. The formation of M-4 from rat microsomes was significantly different between males and females. These metabolites produced by microsomes correspond well to the major oxidized metabolites previously observed in vivo in the serum and the urine following the oral administration of DM to rats, dogs, monkeys and humans.

EVIDENCE FOR THE EXISTENCE AND LOCATION OF A DERMAL RESERVOIR FOR BENZYL ACETATE.

A.Garnett, S.A.Hotchkiss & J.Caldwell. Dept. Pharmacology &Toxicology, St.Marys Hospital Medical School, Imperial College , London. W2 1PG

Consideration of systemic exposure via dermal penetration is rapidly becoming an important facet in compound safety evaluation. The possible formation of a depot within the skin means that systemic exposure could occur even after topical application of the compound has ceased. Full thickness male F344 rat skin and fresh human (surgical) tissue were used to assess the penetration of benzyl acetate (BA) a widely used fragrance agent, and its formation of a cutaneous reservoir. ^{14}C-BA (16.1mg/cm^2) was applied to the epidermal surface of the tissues in an in vitro flow-through diffusion system . At various times up to 6h the skin was swabbed with gauze soaked in ethanol to remove unabsorbed compound. Penetration was assessed by quantifying the radioactivity in the perfusate (Hepes-buffered Hanks balanced salts solution (HHBSS)) which flowed continuously across the underside of the skin at 1.5 ml/h. Residual radioactivity in the skin was assessed by liquid scintillation spectrometery. The location of the reservoir in rat skin was investigated by slicing the skin into 10µm sections parallel to the epidermal surface with a microtome and by serial tape stripping. Radioactivity was counted in both skin sections and tape strips. BA was rapidly absorbed into rat skin reaching a maximum of 66% at 1h which was maintained for 6h. Human skin absorbed the compound more slowly to reach between 16-21% of the applied dose at 1h which was also maintained for 6h. Sectioning the rat skin demonstrated that the majority of the radioactivity applied was found in the upper 30µm of the epidermis. Tape stripping confirmed that the reservoir is likely to exist within the upper layers of cells of the stratum corneum since ca.18% of the dose was located in this region. This suggests the presence of a reservoir in the stratum corneum into which BA rapidly partitions after topical application, and from which it is slowly released into the systemic circulation.

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CHARACTERIZATION OF 3 HUMAN FLAVIN CONTAINING MONOOXYGENASES

Rodolfo Gasser, Ronald E. Tynes° and Urs A. Meyer, Dept. of Pharmacology, Biocenter of the University of Basel, CH-4056 Basel, Switzerland; ° Biopharmaceutical Department, Drug Safety, Sandoz Ltd., CH-4002 Basel Switzerland.

The microsomal flavin-containing monooxygenase (FMO, EC 1.14.13.8) catalyses the oxidation of a wide variety of drugs, pesticides, industrial chemicals and plant and animal components, such as nicotine and trimethylamine. As a first step towards the elucidation of the importance and function of the FMO enzymes in humans we employed molecular biology techniques to identify and characterize 3 forms of FMO in human tissues.

A FMO species homolog to the pig and rabbit hepatic FMO forms (1) was found to be expressed mostly in human kidney and not in human liver. DNA sequence analysis showed a great degree of identity (87%) of this FMO, named Form 1, to both the pig and the rabbit liver FMO protein. The virtual absence of this form in human liver precludes the involment of this FMO species in the genetic disease, trimethylaminuria.

Form 2 FMO is expressed at low levels in human lung and liver and is 83% homologous to rabbit pulmonary FMO (2).

A putative third FMO species, Form 3, was isolated from human liver. It has not been previously characterized and accounts for the majority of FMO expressed in human liver. The later form is thought to be involved in trimethylaminuria.

All 3 forms share app. 60-70 % homology between each other and are products of single genes.

In conclusion, the distribution and expression of FMOs in humans differs considerably from the animal species previously described and reflects the differing catalytic activities attributed to FMOs in human tissues.

1. Gasser, R., Tynes, R.E., Lawton, M.P., Korsmeyer, K.K., Ziegler, D.M., and Philpot, R.M. *Biochemistry* 29, 119 (1990)
2. Lawton, M.P., Gasser, R., Tynes, R.E., Hodgson, E., and Philpot, R.M. *J.Biol.Chem*, 5855-5861 (1990)

SELECTIVE, GROWTH-DEPENDENT INDUCTION OF GST-Yp BY CARCINOGENS IN CULTURED HEPATOCYTES

Rolf Gebhardt, Physiologisch-chemisches Institut, D-7400 Tübingen, FRG

The demand for rapid and reliable screening systems for potential carcinogens prompted us to study the effects of carcinogens and non-carcinogens on the expression of different glutathione-S-transferase (GST isozymes in cultured rat hepatocytes. Hepatocytes cultured at high cell density (non-proliferating) in the presence of dexamethasone maintained their phenotypic spectrum of GST and did not express the preneoplastic marker enzyme GST-Yp irrespective whether these cells are exposed to carcinogens or not (Cell Biol Toxicol 6:365-378, 1990). However, if hepatocytes are cultured at low cell density (or are stimulated by EGF to grow) GST-Yp is induced by several carcinogens (AAF, DEN, 3-MC, 4-aminobiphenyl, etc.) but not by non-carcinogens like phenobarbital, nicotinamide as well as by peroxisome proliferators which also do not induce GST-Yp in vivo. Addition of hydroxyurea to proliferation-competent cells blocked cell growth and impaired the carcinogen-induced increase in GST-Yp. In contrast, phenotypic isozymes (Ya and Yb) were affected by both groups of chemicals and responded similar under all culture conditions.

These results indicate that the selective induction of GST-Yp by carcinogens is growth-dependent explaining at least partly the suitability of this enzyme as a preneoplastic marker. It is suggested that GST-Yp may also prove useful as a carcinogenic marker in hepatocyte cultures, if these are maintained under specific growth-promoting conditions.

SEX AND AGE DEPENDENT DIFFERENCES IN THE HEPATIC SULFOTRANSFERASE ACTIVITY.

R.A.H.J. Gilissen, G.J. Mulder and J.H.N. Meerman.Division of Toxicology, Center for Bio-Pharmaceutical Sciences, University of Leiden, Leiden, The Netherlands.

Various toxic compounds are converted by sulfotransferases to unstable, reactive metabolites that may bind covalently to cellular macromolecules. The expression of some of the sulfotransferases involved is under hormonal control. For instance, sulfation of the hydroxamic acid N-hydroxy-2-acetylaminofluorene (N-OH-2AAF) in the liver (by aryl sulfotransferase IV) is higher in adult males than in adult females (Debaun et al, Cancer Res,50,577-595,1970), whereas sulfation of 7-hydroxymethyl-12-methylbenz[a]antracene (7-OH-HMBA) (by a hydroxysteroid sulfotransferase) is higher in females than in males (Suhr et al.,Biochem.Pharmacol,41,213-221,1991).

In order to investigate whether sulfation of other compounds may also differ with sex and age, we measured sulfation activity towards various substrates in liver cytosols of adult males, females and preweanling rats, using a sulfation assay that measures substrate dependent formation of PAP by HPLC (Duffel et al.,Anal.Biochem,183,320-324,1989). Similar sex and age related differences in sulfation activity towards various hydroxamic acids (N-OH-2AAF, N-OH-2AAP, N-OH-4FAABP and N-OH-4AABP) were found : adult male sulfation was higher (4 to 6 times) than adult female and preweanling sulfation activity. Sulfation activity towards various hydroxylamines (N-OH-2AF, N-OH-2AP, N-OH-4FABP and N-OH-4ABP) did not vary with sex or age. However, various other compounds (1'-hydroxy-safrole, 1'-hydroxy-allylbenzene, 1,2,3,4-tetrahydro-1-naphthol) were also sulfated in a sex and age depended manner : adult male sulfation activity was higher (2 to 4 times) than female and preweanling rat sulfation activity. Sulfation activity towards various hydroxymethyl polyaromatic hydrocarbons (9-hydroxyfluorene, 9-hydroxymethylantracene and 4-hydroxymethyl-biphenyl) were also measured. Only sulfation activity towards 9-hydroxymethylanthracene displayed sex and age related differences : sulfation in preweanling rats was twice as high as in adult female rats. Adult male rats had no hepatic sulfation activity towards this compound.

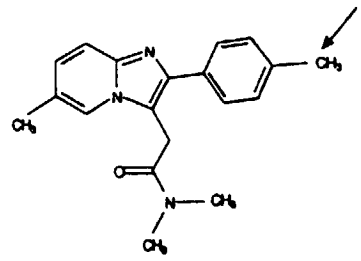
In conclusion : the observed sex and age differences in hepatic sulfation activity confirm previously reported sex and age differences in sulfation activity.

IN VITRO AND *IN VIVO* METABOLISM OF ZOLPIDEM IN THREE ANIMAL SPECIES AND IN MAN

Gérard Gillet, Jean-Paul Thénot, Paolo L. Morselli, Synthélabo-Recherche (L.E.R.S.), 23-25, av. Morane Saulnier, 92360 Meudon-La-Forêt, France

The metabolism of zolpidem was studied *in vitro* after incubation with mouse, rat and monkey hepatic microsomes. In these animal species Km and Vmax values were 247.7, 92.5, 157 µM and 46.9, 6.6, 31.9 nmole.min $^{-1}$, respectively. Thus, the intrinsic clearance is lower in the rat (0.071 ml.min $^{-1}$) than in the mouse and the monkey (about 0.2 ml.min^{-1}). The primary metabolites with microsomes are alcohols (arising from hydroxylation of the methyl groups) which are further oxidized by cytosolic enzymes; so the end metabolites in urine are carboxylic acids. Amongst the 3 possible methyl groups hydroxylation of the one attached to the benzene ring is by-far the predominant one.

This is confirmed *in vitro* by the partial metabolic clearance to the alcohol and *in vivo* by the metabolic profiles in urine. The latter show that the corresponding carboxylic acid accounts for between 72 to 86 % of the urinary metabolites.



THE ROLE OF CYTOCHROME P450 VERSUS NADPH-CYTOCHROME P450 REDUCTASE IN THE REDUCTION OF DUROQUINONE

A.R. Goeptar, J.M. te Koppele, L.M.H Koymans, J.M. Piqué and N.P.E. Vermeulen, Department of Pharmacochemistry, Division of Molecular Toxicology, Faculty of Chemistry, Free University, De Boelelaan 1083, Amsterdam, The Netherlands.

Reductive bioactivation of quinones in mammalian liver has been attributed to NADPH-cytochrome P450 reductase (RED). Recently we found that cytochrome P450 (P450) is also involved in the reduction of duroquinone (2,3,5,6-tetramethylbenzoquinone, DQ) [1]. In phenobarbital-induced rat liver microsomes, (employing SK&F 525-A), and in reconstituted systems of purified P450-IIB1 and RED, we were able to demonstrate P450-mediated reduction of DQ to the semiquinone radical (DSQ) by electron spin resonace (ESR).

The present study was conducted to establish the contribution of RED and P450-IIB1 in the enzymatic reduction of DQ. Therefore, initial rates of NADPH oxidation were determined as a measure of quinone metabolism [2] in reconstituted systems containing varying amounts of RED and P450-IIB1. As expected, RED (70 μM) was able to reduce DQ at low rates in the absence of P450-IIB1. Reconstitution in the presence of increasing amounts of P450-IIB1 resulted in increasing rates of NADPH oxidation. From these data it was calculated that NADPH oxidation occured at a rate of 26.3 pmol NADPH/nmol P450-IIB1/min. Similarly, experiments with varying amounts of RED (and 70 μM P450-IIB1) showed NADPH oxidation by RED at a rate of 30.6 pmol NADPH/nmol RED/min. Thus it was concluded that P450-IIB1 and RED contribute 0.9:1 to the reduction of DQ.

Interestingly, all experiments performed at a 1:1 molar ratio of P450-IIB1 and RED consistently resulted in a 4:1 contribution of P450-IIB1 in DQ reduction as compared to RED (using ESR and NADPH oxidation) [1]. Inasmuch as the present study unambigously resulted in a ratio of 0.9:1, it was suggested that P450-IIB1 modulates the efficiency of RED. Evidence will be presented that this is indeed the case; quinone reduction by RED is enhanced 4-fold by P450-IIB1.

[1] Goeptar et al. Eur. J. Pharmacol. 183, 1990, 1363-1364.
[2] Powis and Appel. Biochem. Pharmacol. 31, 2745-2753, 1982.

INTERINDIVIDUAL DIFFERENCES IN METABOLISM AND STEREOCHEMICAL EFFECTS INFLUENCE BIOMONITORING OF STYRENE

H.W. Goergens*, M. Linscheid**, M. Korn***, K. Golka, H. Peter, E. Hallier
*Arbeitsmed. Zentrum, TÜV Rheinl., Friedr.-Engels-Allee 346, D-5600 Wuppertal 2
** Institut für Spektrochemie, Bunsen-Kirchhoff-Str. 2, D-4600 Dortmund
***Inst. f. Arbeits- und Sozialmedizin, Wilhelmstr. 27, D-7400 Tübingen 1
Inst. f. Arbeitsphysiologie , Ardeystr. 67, D-4600 Dortmund, FRG

A possible carcinogenicity of sytrene is at present under discussion. Mandelic acid and phenylglyoxylic acid are the biomonitors of exposure most commonly recommended. The implementation of adducts with blood proteins or lymphocyte DNA has recently been proposed as an alternative.

Blood and urine samples of 20 workers occupationally exposed to an average of 50 ppm styrene were collected. The lymphocytes were isolated by density centrifugation. They were either cultivated for the determination of SCE´s or for the extraction of DNA. Hemoglobin and serum albumin were isolated for the determination of adducts. From the urine samples, the enantiomers of mandelic acid were isolated and analyzed on a chiral GC column. The mercapturic acids of styrene oxide were analyzed in the urine by thin layer chromatography.

Only a part of the workers exposed to styrene excreted the mercapturic acids of styrene oxide, possibly due to an enzyme polymorphism of the liver glutathione transferase μ previously described by Warholm et al. (Biochem. 22, 3610, 1983). The ratio of R- and S-mandelic acid excreted in the urine varied interindividually from 1:1 to 1:4. R-styrene oxide has been shown to be a stronger mutagen than the S-form in the Ames-test by Pagano et al (Environ: Mut. 4, 575, 1982). This coincides with the formation of enantioselectively different styrene oxide adducts in DNA as recently shown by Seiler (Mut. Res. 245, 165, 1990).

The rate of SCE in the lymphocytes of the exposed workerswas significantly enhanced. In addition, enantioselectively different adducts in human blood proteins were determined by FAB (fast atom bombardment) mass spectrometry.

A comparative evaluation of the different biomonitors of exposure to styrene shows that the excretion of urinary products is subjected to interindividual differences in metabolism such as enzyme polymorphism. Adducts with different blood macromolecules could be superior for biomonitoring because they reflect the biological activity of the ultimate reactive metabolite styrene oxide.

AVAILABILITY OF COENZYME A AND GLYCINE LIMITS GLYCINE CONJUGATION OF BENZOIC ACID *IN VIVO*

Zoltán Gregus[1], Tibor Fekete[1], Ferenc Varga[1] and Curtis D. Klaassen[2], [1]Department of Pharmacology, University Medical School, Pécs, Hungary and [2]Department of Pharmacology, Toxicology and Therapeutics, University of Kansas Medical Center, Kansas City, KS.

Using benzoic acid as substrate, we tested the hypothesis that capacity limitation of glycine conjugation *in vivo* is due to substrate-induced depletion of hepatic cosubstrates (i.e., ATP, coenzyme A and/or glycine) utilized in the reaction. Benzoylglycine formation was investigated by following disappearance of benzoic acid from blood and appearance of benzoylglycine in blood and urine after administration of sodium benzoate (0.2-2 mmol/kg, iv) to anaesthetized rats whose urine formation was stimulated by mannitol administratation. Capacity limitation of glycine conjugation is indicated by (1) a gradual dose-dependent reduction of benzoate blood clearance from 39 ml/min/kg after 0.2 mmol/kg benzoate to 3.7 ml/min/kg after 2 mmol/kg, and (2) the tendency to attain maximal blood levels and urinary excretion rates of benzoylglycine after administration of 0.5-1 mmol/kg benzoate. The urinary excretion rate of endogenously formed benzoylglycine reflects the rate of its formation because the maximal urinary excretion rate of benzoylglycine after benzoylglycine administration exceeded the maximal excretion rate of endogenously formed benzoylglycine (approximately 5 μmol/kg/min) five fold. Benzoate depleted hepatic glycine (60%) and coenzyme A (86%) in a dose-dependent fashion, however, it did not change ATP levels in liver. The pattern of this dose-dependent cosubstrate depletion suggests that benzoate primarily causes consumption of hepatic glycine which leads to marked depletion of hepatic coenzyme A after a high substrate dosage. Thus, these observations indicate that capacity limited glycine conjugation may be due to limited availability of glycine and coenzyme A for the conjugation process.

SPECIES DIFFERENCES IN INDUCTION OF HEPATIC AND EXTRAHEPATIC CYTOCHROMES P-450IA and IIB BY MIXED-TYPE INDUCERS

Alevtina Yu. Grishanova, Lyudmila F. Gulyaeva and Vyacheslav V. Lyakhovich, Institute of Clinical and Experimental Medicine, Novosibirsk, USSR

The effects of treatment with Aroclor 1254 (Ar) and hexachlorobenzene (HCB) on the liver, lung and kidney monooxygenase activities, barbiturate-and hydrocarbone-inducible cytochrome P-450 forms were evaluated in adult male CBA mice and Wistar rats. Both P-450 isozymes (IA1 and IA2) are indu- ced in the lung after treatment of the rats with Ar or HCB, whereas in the lung of CBA mice only P-450IA1 is induced by these compounds. In rat kidney Ar and HCB caused induction of P-450IA1 only compared to P-450IA1 and IA2 in CBA mice kid-ney. As for P-450IIB gene family, Ar and HCB indu-ced two isozymes IIB1 and IIB2 in rat lung. Ar-induced expression of one more P-450IIB gene member distinct from IIB1 and IIB2 in rat lung and liver was detected. P-450IIB1 was observed in the kidney of rats treated with Ar but not HCB. P-450 IIB was found in the lung of Ar- and HCB-treated CBA mice and was not detected in the kidney. Alkoxyresorufin-O-dealkylase activities as probes for P-450IA and IIB isozymes were evaluated in all investigated microsome patterns. These data showed different potency of PB- and MC-type induction by Ar and HCB in extrahepatic tissues.The differences in the induction of cytochromes P-450IA and IIB suggest that these enzymes exhibit species-, tissue- and inducer-dependent specificity.

DISPOSITION OF HEXOBARBITAL ENANTIOMERS AND ANTIPYRINE IN AGEING DIETARY RESTRICTED RATS.

Kees Groen, Adriaan Brouwer, Johan M. te Koppele, Evelien J. Jansen, Gerrit Schijff, Douwe D. Breimer, and Cornelis F.A. van Bezooijen. TNO Institute for Ageing and Vascular Research, Zernikedreef 9, 2333 CK Leiden, The Netherlands.

The influence of dietary restriction (DR) on the metabolism of hexobarbital (HB) enantiomers and antipyrine (AP) was studied in a longitudinal experiment using female Brown Norway rats. A 40% reduction in food intake resulted in a increased life span in these rats. 20% of the *ad libitum* (AL) fed animals survived till 33 months, whereas 85% of the DR rats survived till that age. At the age of 4, 13, and 25 months, the rats received an oral dose of HB and AP. Plasma clearance (Cl_s) of S-HB, R-HB, and AP expressed per kg body weight was significantly higher in DR rats at all ages when compared with AL rats. In both groups of rats, Cl_s was five to six times higher for S-HB than for R-HB. For both HB enantiomers, $T_{1/2}$ was not different between AL and DR rats at any age. $T_{1/2}$ of AP was significantly longer in AL rats at the age of 25 months when compared with DR rats. However, the differences in AP kinetics as presented here are not observed when AP is given simultaneously with theophylline. Probably, the central depressant effect of HB as observed in the AL rats only, is partly responsible for the observed differences. From these data, it can be concluded that dietary restriction has no negative effect on the drug metabolising activity of P-450.

DISPOSITION OF SOLANINE IN RATS AND IN HAMSTERS

Kees Groen, Gerrit J.A. Speijers, Peter Besamusca, Diana P.K.H. Pereboom, Piet K. Beekhof, and Henk J.G.M. Derks. Nat. Inst. of Public Health and Environmental Protection, P.O. Box 1, 3720 BA Bilthoven, The Netherlands.

Solanine is a toxic glycosidic alkaloid present in the common potato (*Solanum tuberosum*). The pesticide properties of the glycosidic alkaloids are used in new potato cultivars with relatively high alkaloid concentrations. In an experiment in rats and hamsters, the disposition of ^{3}H-solanine was studied after oral and after intravenous dosage, in order to decide which species to use for risk assessment studies. The elimination half life was about 30 hours in both species. However, after oral administration the elimination phase of solanine from plasma is much faster in the rat than in the hamster ($T_{1/2}$ 7.8 vs 23 h). After oral administration in the rat, the elimination was faster than after i.v. administration, whereas in the hamster there was no difference. The bio-availability of unchanged solanine after oral administration was 1.8% in the rat and 7.0% in the hamster. After i.v. dosing, 25% of the dose was recovered as total activity in urine within 172 hours in both rat and hamster. However, after oral dosing the recovery in urine was about 10% in the hamster and only 3% in the rat.
These data indicate that the hamster is a better model for risk assessment studies than the rat.